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TITLE: The Role of mDia1 in the Aberrant Innate Immune Signaling in del(5q) Myelodysplastic Syndromes

PRINCIPAL INVESTIGATOR: Dr. Peng Ji

CONTRACTING ORGANIZATION: Northwestern University
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14. ABSTRACT
Myelodysplastic syndromes (MDS) are a group of diseases affecting bone marrow and blood with an increased risk of developing acute leukemia. fMany genomic abnormalities are associated with MDS with deletion of chromosome 5q being the most common. Our recently published work demonstrated that loss of mDia1, a protein with its encoding genes located at chromosome 5, led to the activation of the innate immune response through an aberrant overexpression of CD14 on granulocytes, which accelerate the development of MDS in mDia1 deficient mice. Based on this study, we hypothesis that CD14 induced abnormal immune response is critical in the pathogenesis of MDS. In the past grant reporting cycle, we have successfully demonstrated the significance of in vivo damage associated molecular patterns (DAMPs) in the pathogenesis of MDS. Significant achievements were also obtained in other major goals. We demonstrated the efficacies of CD14, AP1, and SP1 inhibitors in the downregulation of CD14 in granulocytes. CD14 and mDia1 double knockout are also under breeding. There are no major changes of the experimental designs.

15. SUBJECT TERMS
Myelodysplastic syndromes, del(5q), mDia1, CD14, innate immune signaling, TLR4, DAMPs, PAMPs, inhibitors, mouse model

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INTRODUCTION

The overall goal of this project is to determine the role of mDia1 in the innate immune signaling and the pathogenesis of del(5q) MDS. Deletion of chromosome 5 long arm (del(5q)) is the most common cytogenetic abnormality in patients with myelodysplastic syndromes (MDS). We recently discovered that CD14 was aberrantly overexpressed on granulocytes of mice with loss of mDia1, whose encoding gene is located at 5q31. This led to a hypersensitive innate immune response to the stimuli of lipopolysaccharide (LPS) in mDia1 heterozygous and knockout mice. Importantly, chronic stimulation of these mice with LPS accelerated the development of MDS that normally occurs in aged mDia1 deficient mice. CD14 was also found overexpressed on granulocytes of patients with del(5q) MDS. Based on these published results, we hypothesize that mDia1 deficiency induced aberrant innate immune signaling, through CD14 and its co-receptor TLR4, is critical for the pathogenesis of del(5q) MDS. To test this hypothesis, we proposed three specific aims. In aim 1, we will determine how damage associated molecular patterns (DAMPs), as the in vivo ligands for CD14/TLR4, are involved in the hypersensitive innate immune response and development of MDS in mDia1 deficient mice. In aim 2, we will determine how CD14 is involved in the development of MDS in mDia1 deficient mice using CD14/TLR4 inhibitors and a novel CD14/mDia1 double knockout mouse model. Aim 3 will focus on the mechanism of CD14 upregulation with loss of mDia1. The possible role of transcriptional repression of CD14 in rescuing MDS in mDia1 deficient mice will also be determined. Successful accomplishment of our proposed project is important for the understanding of the pathogenesis of aberrant innate immune responses in del(5q) MDS. The new CD14/mDia1 double knockout mouse model and novel concept of using pharmacologic inhibitors to target CD14/TLR4 would also greatly help the development of novel immunotherapeutic management for MDS.

KEYWORDS
Myelodysplastic syndromes, del(5q), mDia1, CD14, innate immune signaling, TLR4, DAMPs, PAMPs, inhibitors, mouse model.

ACCOMPLISHMENTS

What were the major goals of the project?
The major goals of the project are listed below. The completion dates or the percentage of completion are noted.

- Major goal 1: Determine the role of DAMPs in the hypersensitized innate immune response in mDia1 deficient granulocytes in vitro.
  - Completed in August 2016.
- Major goal 2: Injection of DAMPs to mDia1 heterozygous and knockout mice to determine whether they can induce phenotypes mimicking del(5q) MDS.
  - Percentage of completion: 50%
- Major goal 3: Determine the effect of CD14 inhibitors on the response of mDia1 deficient mice treated with LPS or DAMPs.
  - Percentage of completion: 50%
- Major goal 4: Determine whether knockout of CD14 would rescue the hypersensitized innate immune responses in mDia1 deficient mice.
  - Percentage of completion: 30%
- Major goal 5: Determine the transcription factors that are responsible for CD14 upregulation during granulocytic differentiation in vitro in mDia1 deficient Gr1/Mac1 double positive cells.
  - Percentage of completion: 70%
- Major goal 6: Determine whether mDia1 directly interacts with AP1 or SP1 to influence their transcriptional regulation of CD14.
  - Percentage of completion: 30%
- Major goal 7: test whether inhibition of CD14 transcription pharmacologically would abolish CD14 overexpression and myelodysplasia.
  - Percentage of completion: 20%

What was accomplished under these goals?
The accomplished work of each major goal is described below.
<u>Major goal 1: Determine the role of DAMPs in the hypersensitized innate immune response in mDia1 deficient granulocytes in vitro.</u>

This goal is completed. We have already prepared a manuscript that is ready to be submitted. **The manuscript is attached in the appendices**. In this manuscript, we generated a mDia1 and miR-146a double knockout mouse model which exhibited more severe MDS phenotypes than mDia1 or miR-146a single knockout mice. The rationale of using miR-146a knockout mouse model in this study is that we can better evaluate the role of mDia1 in a more pathologically relevant background since miR-146a is also located on chromosome 5q. In addition, miR-146a is also involved in the innate immune signaling. Loss of miR-146a in mice causes a similar hyperactivated innate immune response similar to that in mDia1 knockout mice.

Given the significance of the bone marrow microenvironment in the development of anemia, we first examined the levels of proinflammatory cytokines in old mDia1/miR-

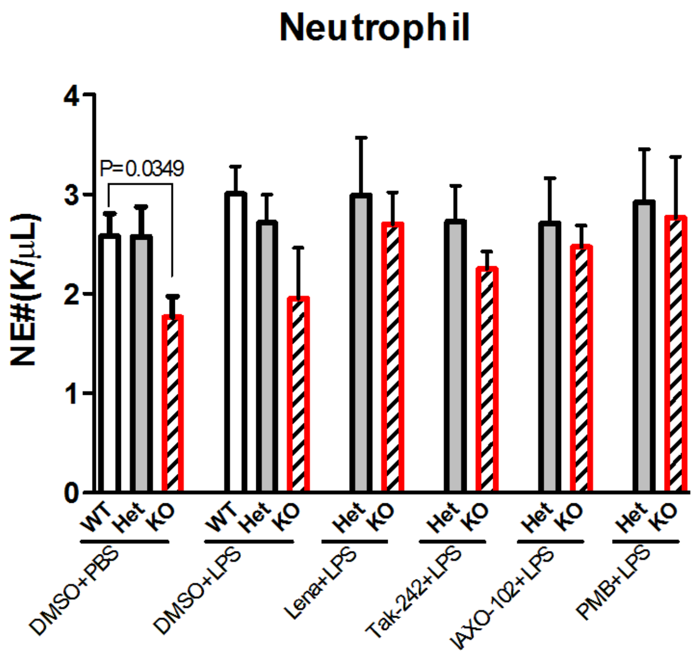
146a double knockout mice and littermate controls. As expected, serum levels of both IL-6 and TNFα were significantly increased in old double knockout mice compared to age-matched double wild type and single knockout controls (Figure 6A in the manuscript). The ageing bone marrow microenvironment is composed of increasing amounts of DAMPs, which are potent inducers of proinflammatory cytokines production. We previously demonstrated that mDia1 deficient Gr1/Mac1 positive granulocytes over-produced TNFα and IL-6 when treated with LPS. A similar phenotype was also observed in miR-146a knockout mice. To analyze whether treatment of DAMPs could also induce the over-production of proinflammatory cytokines in old double knockout mice in vitro, we purified Gr1 and Mac1 double positive granulocytes from the bone marrow and spleen of young double knockout mice and littermate controls. These cells were treated with DAMPs prepared through repetitive freeze thaw cycles of wild-type bone marrow cells. Similar to LPS, treatment with DAMPs induced over-production of TNF-α and IL-6 in the bone marrow- and spleen-derived myeloid cells from each group of mice, with levels particularly high in double knockout cells (Figure 6B in the manuscript). TNFα and IL-6 levels were also dramatically higher in CD3ε positive T cells from the spleen of double knockout mice compared to the controls (Figure 6C in the manuscript). These results indicate that bone marrow microenvironment-mediated ineffective erythropoiesis in double knockout mice could be related to the over-secretion of proinflammatory cytokines.

Major goal 2: Injection of DAMPs to mDia1 heterozygous and knockout mice to determine whether they can induce phenotypes mimicking del(5q) MDS.

We have started to inject DAMPs in mDia1 heterozygous and knockout mice. The same injection experiment was also performed in mDia1/miR-146a double knockout mice. We first tried several different forms of DAMPs for the in vivo injection. In the in vitro experiments described in major goal 1, we used bone marrow cell lysate from repeated freeze-thaw cycle as DAMPs, which include a mixture of various components including proteins and nucleate acids. In vitro experiments as described above showed potent abilities of these DAMPs to induce aberrant proinflammatory cytokine production in neutrophils and T-lymphocytes.

We first performed preliminary experiments using DAMPs from bone marrow cell lysates to inject the wild type mice. The wild type mice did not exhibit detectable abnormalities in complete blood count or appearance three weeks after injection. We plan to start the injection in wild type mice more than one year old to determine whether aging environment would accelerate the inflammation induced by DAMPs. We also plan to inject DAMPs in mDia1/miR-146a double knockout mice since these mice are more sensitive to the stimulation of the innate immune signaling.

Major goal 3: Determine the effect of CD14 inhibitors on the response of mDia1 deficient mice treated with LPS or DAMPs.



We have performed preliminary studies on this major goal. Specifically, mDia1 wide type (WT), heterozygous (Het) and knockout (KO) mice were intraperitoneally injected with 1mg/kg Lipopolysaccharide (LPS) simultaneously with various inhibitor as indicated in the Figure every 2 weeks for 5 consecutive months. Lena indicates lenalidomide (10mg/kg), which is commonly used to treat del(5q) MDS. Tak-242 (3mg/kg) is a commercially available NF-kB inhibitor. IAXO-102 (1mg/kg) is a commercially available CD14 inhibitor. PMB is Polymyxin B (2000U/kg), antibiotics against Gram-negative bacteria. Complete blood counts were performed after the last injection and neutrophil counts were shown in the Figure. As shown, mDia1 KO mice showed prominent neutropenia. LPS treatment increased neutrophil counts in wild type mice, which was not present in mDia1 Het and KO mice. As we expected, lenalidomide, NF-kB inhibitor, CD14 inhibitor, and antibiotics all rescued neutrophil count in mDia1 knockout mice. These data suggest that CD14 inhibitor plays a similar role as other commonly used compounds for treating cytopenia, thus providing basis for the possible future development of CD14 inhibitors to treat MDS.

We will repeat these experiments to make sure they are reproducible. Other CD14 inhibitors will also be tested to determine it is a general phenotype with the inhibition of CD14.

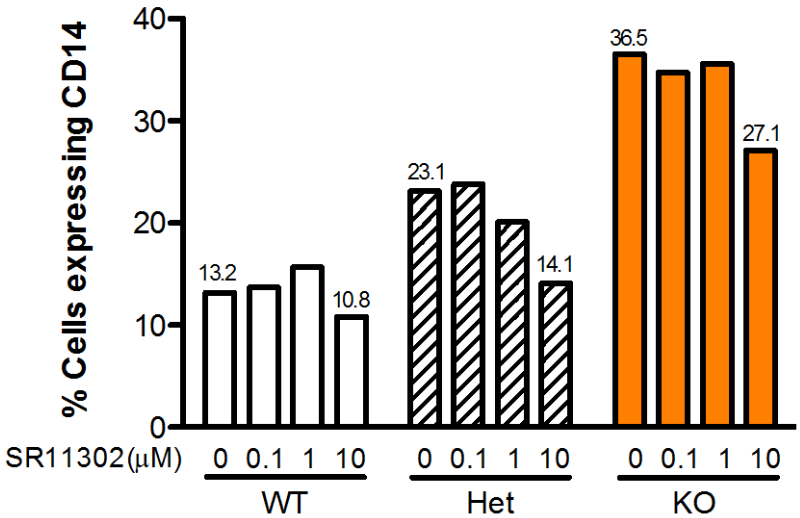
<u>Major goal 4: Determine whether knockout of CD14 would rescue the hypersensitized innate immune responses in mDia1 deficient mice.</u>

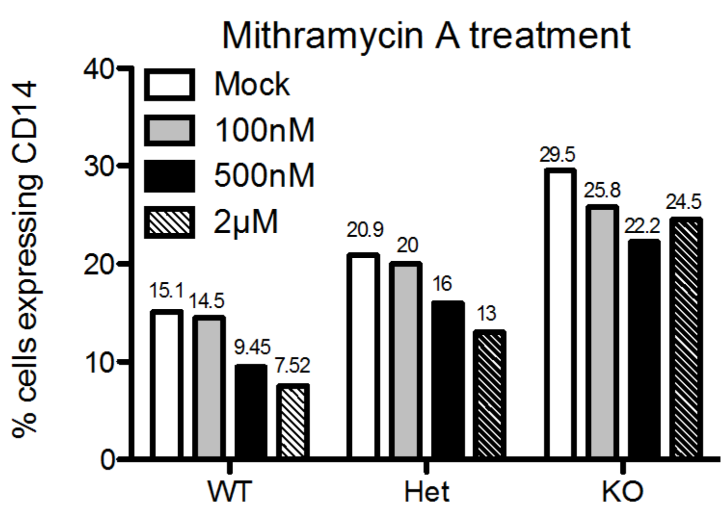
We have purchased CD14 knockout mice from Jackson laboratory. These mice have been crossed with mDia1 knockout mice. The breeding is ongoing and the results will be reported in the future.

<u>Major goal 5: Determine the transcription factors that are responsible for CD14 upregulation during granulocytic differentiation in vitro in mDia1 deficient Gr1/Mac1 double positive cells.</u>

We have performed preliminary experiments using inhibitors of AP1 and SP1. As shown below in the figures, flow cytometry of cell surface CD14 levels on lineage negative cells cultured in vitro were performed. Specifically, we purified lineage negative bone marrow progenitor cells from mDia1 wild type (WT), heterozygous (Het), and knockout (KO) mice. The cells were cultured for 3 days in GM-CSF medium with indicated amount of SR11302 (AP1 inhibitor) or Mithramycin A (SP1 inhibitor). The cells were harvested on day 3 and followed by cell surface CD14 analysis by flow cytometry.

The data below show that both AP1 and SP1 inhibitor affected CD14 levels in mDia1 heterozygous and knockout bone marrow lineage negative cells differentiating to myeloid cells. These experiments establish the basis for the next step mechanistic study. We will repeat the experiments to obtain statistic significance. We will also use shRNA to knockdown AP1 and SP1 to circumvent the nonspecific inhibition from the using of the inhibitors.





Major goal 6: Determine whether mDia1 directly interacts with AP1 or SP1 to influence their transcriptional regulation of CD14.

We performed co-immunoprecipitation experiments using HET293T cells transfected with AP1 or SP1, together with mDia1. We encountered some technical problems with the antibodies to AP1 and SP1. Strong background bands were detected. We plan to change the antibodies, or use different cell lines to perform transfection, to reduce the background signal. The follow up experiments will be reported in the next grant report period.

Major goal 7: test whether inhibition of CD14 transcription pharmacologically would abolish CD14 overexpression and myelodysplasia.

As indicated by the figures in major goal 5, inhibition of AP1 or SP1 reduced CD14 expression level in mature myeloid cells. These data indicate that inhibition of CD14 transcription pharmacologically using AP1 or SP1 inhibitor will abolish CD14 overexpression in vitro. We plan to perform in vivo injection of AP1 or SP1 inhibitors in mDia1 heterozygous and knockout mice to determine whether CD14 levels will reduce in granulocytes. We will also determine whether the inhibitors will abolish myelodysplasia after chronic injection.

What opportunities for training and professional development has the project provided?
During the past year of the funding period, I have a scientific advisory committee including Drs. John Crispino and William Muller, which meet twice every year. In the past years, it has been extremely helpful to provide me guidance scientifically and in professional development. We will also have a monthly joint hematopoiesis group meeting with members from the laboratories of Drs. John Crispino, Jonathan Licht, Seth Corey, and Elizabeth Eklund in the Division of Hematology/Oncology at Northwestern University. The finished part of the project has been selected for presentation at the annual American Society for Hematology (ASH) meeting.

How were the results disseminated to communities of interest?
Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

- Major goal 1: Accomplished

- Major goal 2: We plan to start the injection in wild type mice more than one year old to determine whether aging environment would accelerate the inflammation induced by DAMPs. We also plan to inject DAMPs in mDia1/miR-146a double knockout mice since these mice are more sensitive to the stimulation of the innate immune signaling.

- Major goal 3: We will the experiment presented above under major goal 3 to make sure they are reproducible. Other CD14 inhibitors will also be tested to determine it is a general phenotype with the inhibition of CD14.

- Major goal 4: The CD14/mDia1 double knockout mice are breeding. We expect to be able to report their phenotypes in the next reporting period.

- Major goal 5: We will repeat the AP1 and SP1 inhibitor experiments to obtain statistical significance. We will also use shRNA to knockdown AP1 and SP1 to circumvent the nonspecific inhibition from the using of the inhibitors.

- Major goal 6: We plan to change the antibodies, or use different cell lines to perform transfection, to reduce the background signal. The follow up experiments will be reported in the next grant report period.

- Major goal 7: We plan to perform in vivo injection of AP1 or SP1 inhibitors in mDia1 heterozygous and knockout mice to determine whether CD14 levels will reduce in granulocytes. We will also determine whether the inhibitors will abolish myelodysplasia after chronic injection.

IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Our findings demonstrate that ageing related bone marrow changes can affect the development of myelodysplastic syndromes (MDS). It also has impact in the military field in which military personnel with increased exposure to external stimulations of their innate immune signals may have increased risk of developing MDS.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

CHANGES/PROBLEMS

Nothing to Report

PRODUCTS

Nothing to Report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Peng Ji
Project Role:	PI
Nearest Person Month Worked:	4
Contribution to Project:	Dr. Ji performed the overall design and analysis of the results, writing of the manuscript.
Funding Support:	N/A

Name:	Yang Mei
Project Role:	Post-doctoral Fellow
Nearest Person Month Worked:	8
Contribution to Project:	Dr. Mei performed all the reported experiments and analyzed the data.
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Yes, please see below

Ji, Peng
<u>ACTIVE</u>
Department of Defense W81XWH-15-1-0335 9/15/15 - 9/14/18 3.6 Calendar months
Career Development Award $120,000 directs/year
The role of mDia1 in the aberrant innate immune signaling in del(5q) myelodysplastic syndromes
Role: Principal Investigator.
Aims of the project: to study the role of mDia1 in the innate immune response and how loss of mDia1 contributes to the pathogenesis of myelodysplastic syndromes.
*Award that we're submitting the progress report for – No Change

R01DK102718 (Ji) 4/10/15 - 1/31/20 2.1 Calendar months.
NIH/NIDDK $260,852 directs: yr 2
Title: The roles of pleckstrin-2 as a functional node in erythropoiesis
Aims of the project: To determine the role of pleckstrin-2 in connecting erythropoietin and Rac GTPase signals in the regulation of erythropoiesis.
Role: Principal Investigator
*Was previously pending but has now been awarded

Chicago Biomedical Consortium Catalyst Grant 3/1/15 - 2/28/17 0.24 Calendar months
Nuclear opening and histone release in mammalian terminal erythropoiesis
Role: Principal Investigator. $60,000 directs/year
Aims of the project: To determine the role of caspase-3 in nuclear opening formation and histone release in both human and mouse models of terminal erythropoiesis.
*No Change

The following grant has ended:
K99/R00 HL102154-04 (Ji) 8/17/12 - 5/31/15 9.0 Calendar months
NIH/NHLBI $149,822 directs/year

Analysis of mDia formins in hematopoietic stem cell engraftment and migration
Role: Principal Investigator
Aims of the project: to investigate the role of mDia1 and mDia2 in hematopoietic stem cell engraftment and migration using knockout mouse models.

Crispino, John D.
Role on the reporting project: Mentor

P30CA060553 (Platanias) 08/01/13 - 07/31/18 1.80 Calendar
NIH/NCI $17,970
The Robert H. Lurie Comprehensive Cancer Center
Grant Officer: Dan Rademacher
676 N St. Clair
Suite 1200
Chicago, IL 60611
Telephone: (312)695-1310
Fax: (312) 695-1328
Email: d-rademacher2@northwestern.edu
Cancer Center Support Grant
The goal of this project is to support the activities of the nine established programs and 15 shared resources pertaining to the Northwestern Cancer Research enterprise.
The specific aims of this project are: 1) Conduct and support cancer research and to integrate cancer-related research throughout the university; 2) Coordinate and integrate cancer-related activities of the University including community outreach initiatives; 3) Develop and conduct cancer education programs; 4) Promote and participate in state-of-the-are care of cancer patients at the affiliated hospitals of the McGaw Medical Center of Northwestern University and; 5) Develop and implement the initiatives in cancer prevention and control research.
Role: Associate Director for Education & Training 2

R01CA101774 (Crispino) 07/01/03 - 04/30/17 2.40 Calendar
NIH/NCI $190,839
Grant officer: Connie Murphy
8420 Progress Drive, Suite 4082
Frederick, Maryland 21701
Telephone: 301-631-3008
Fax: 301-631-3030
Email: murphco@mail.nih.gov
Mechanisms of Leukemogenesis in Down syndrome
The goals of this project are to: 1) Determine the necessity and sufficiency of selected Hsa21 genes in human AMKL and the murine model of DS-AMKL, 2) Identify the signaling pathways that are activated in AMKL, such as NFAT which lies downstream of DYRK1A, and 3) Determine the contribution of trisomy 21 to B-ALL.
Aim 1: To determine the necessity and sufficiency of selected Hsa21 genes in human DS-AMKL and our murine model of DS-AMKL.
Aim 2: To identify the signaling pathways that are activated in DS-AMKL.
Aim 3: To define the contribution of trisomy 21 to B-ALL.

Award Ltr 6/28/12 (Crispino) 07/01/08 - 06/30/17 0.12 Calendar
Waxman Foundation $20,000
Grant Officer: Amanda Aronson

420 Lexington Ave., Suite 825
New York, NY 10170
Telephone: (646) 398-5274
Fax: (212) 867-4851
Email: aaronson@waxmancancer.org
The GATA-ERG axis in myeloid malignancies.
The goal of this project is to determine how GATA, GATA2, and ERG work together to drive the abnormal growth of a subset of blood cells named myeloid cells. Mouse models and patient data will be used to better understand the process of leukemia development and to design new strategies to treat this disease.
Aim: To study the relationship between GATA1 and ERG in acute leukemia in children with and without Down syndrome.

R01HL112792 (Crispino)	08/01/13 – 04/30/17	2.40 Calendar
NIH/NHLBI	$304,500	

Grant Officer: Kevin Heath
6701 Rockledge Dr.
Bethesda, MD 20892-7926
Telephone: (301) 435-0166
Fax: (301) 451-5462
Email: heathkj@nhlbi.nih.gov
Aberrant Megakaryopoiesis in the Myeloproliferative Neoplasms
The goal of this project will be to identify transcriptional pathways that are dysregulated in PMF megakaryocytes and characterize the causes of aberrant megakaryopoiesis as compared to ET megakaryocytes.
Aim 1: Identify transcriptional pathways that are dysregulated in PMF megakaryocytes
Aim 2: Determine whether small molecule inducers of megakaryocyte differentiation and polyploidization are effective at restraining the proliferation of aberrant megakaryocytes in MPNs
Aim 3: Study the mechanism by which these compounds lead to differentiation and polyploidization of abnormal megakaryocytes

R01CDK101329 (Crispino)	09/18/13 – 07/31/18	1.80 Calendar
NIH/NIDDK	$217,500	

Grant Officer: Carolyn Kofa
6707 Democracy Blvd.
Room 902
Bethesda, MD 20817
Telephone: (301) 594-7687
Fax: (301) 480-3510
Email: kofac@extra.niddk.nih.gov
GATA1 Mutation in Defective Erythropoiesis
The goal of this grant are to define the requirement for the N- terminus of GATA1 in red cell development and study its function in gene regulation.
Aim1: Correlate chromatin occupancy of GATA1s with gene expression defects in primary GATA1s knock-in erythroid progenitor cells to identify key direct target genes that are dysregulated.
Aim 2: Investigate the consequences of the GATA1s mutation on erythroid specification and differentiation.
Aim 3: Determine if loss of the N-terminus reduces the interaction with essential cofactors and in turn affects their chromatin occupancy.

T32CA009560-26 (Green)	07/01/07-06/30/17	0.6 Calendar

NIH/NCI $300,103
Jacquelyn Boudjeda
6120 Executive Blvd., Suite 243-M
Rockville, MD 20852
Phone: (301) 496-1204
Fax: (301) 496-8601
Email: boudjedaj@mail.nih.gov
Carcinogenesis Training Program
The goal of this program is to provide comprehensive, rigorous research training in cancer biology.
Specific Aim: to support nine predoctoral students in the Carcinogenesis Training Program at Northwestern University.
Role: Co-PI

R6480-15 (Crispino) 09/01/15-08/31/17 0.6 Calendar
Leukemia and Lymphoma Society $270,027
Director of Research Administration
The Leukemia & Lymphoma Society
1311 Mamaroneck Avenue, Suite 310
White Plains, New York 10605
Telephone: (914) 821-8301
Fax: (914) 821-3301
Email: researchprograms@lls.org
MLN8237, an Aurora A kinase inhibitor, for the treatment of myeloid malignancies
This is a grant to sponsor a Phase I clinical study of MLN8237 in patients with Acute Megakaryoblastic Leukemia and Primary Myelofibrosis. The grant will also support correlative studies to identify biomarkers of response. This grant has been awarded, but has not started.
Specific aim: To bring our discovery from the bench to the bedside by performing a Phase I study of the aurora A kinase inhibitor MLN8237 in patients with AMKL or PMF.

9001-16 (Crispino) 10/01/15 - 09/30/18 0.60 Calendar
Leukemia & Lymphoma Society $120,012
Director of Research Administration
The Leukemia & Lymphoma Society
1311 Mamaroneck Avenue, Suite 310
White Plains, New York 10605
Telephone: (914) 821-8301
Fax: (914) 821-3301
Email: researchprograms@lls.org
Identification of pathways that promote transformation of the MPNs to AML
The goal of this proposal is to identify tumor suppressor genes whose loss drives the transformation of primary myelofibrosis to acute myeloid leukemia.
Aim 1: Perform a genome-wide screen with a validated CRISPR-Cas9 library to identify genes whose disruption confers in vitro colony re-plating activity to Jak2V617F murine progenitors
Aim 2: Determine the ability of genes identified in the screen to promote leukemic progression of Jak2V617F cells in animal models
Aim 3: Assay whether the identified genes are relevant to human post-MPN AML and evaluate the extent to which they enable primary human MPN CD34+ cells to engraft and promote leukemia in xenograft models.

Research Grant (Crispino) 07/1/16 - 06/30/18 0.12 Calendar
Rally Foundation $50,000

Grants Manager: Erin Seidenburg
5775 Glenridge Drive NE
Building B Suite 370
Atlanta, GA 30328
Telephone: (404) 847-1270
Email: Info@rallyfoundation.org
DYRK1A is a novel target in B cell leukemia
The goals of this grant are to perform pre-clinical studies of novel DYRK1A kinase inhibitors in DS pre-B cell acute lymphoblastic leukemia.
Aim 1: Determine the requirements for DYRK1A in human DS and non-DS B-ALL. We will use both genetic and pharmacologic approaches to determine the requirement for DYRK1A in malignant B-cells. Our goal is to generate pre-clinical data to support further development of DYRK1A inhibitors as a new therapy. Aim 2: Identify the mechanisms by which DYRK1A inhibition causes growth inhibition of ALL cells. We will first study the manner in which the inhibitors impede the growth of ALL cells and assess whether reduced phosphorylation of caspase 9, a known substrate of DYRK1A, contributes to increased cell death of the leukemia cells. Second, we will use an unbiased approach to identify novel substrates of DYRK1A in ALL cells.

Research Grant (Crispino) 10/01/15 – 09/30/17 0.12 Calendar
Heartland Blood Centers $225,000
Director of Research Administration: Andrea Brown
8733 Watertown Plank Road
Milwaukee, WI 53226
Telephone: (414) 937-6355
Email: andrea.brown@bcw.edu
Role of cohesion mutations in Acute Myelogenous Leukemia
The goals of this project are to study the contribution of cohesin mutations to hematopoiesis and AML, examine the connection between loss cohesin or CTCF and trisomy 21, and to elucidate the molecular mechanism for cohesin and CTCF involvement in progression to DS-AMKL
Aim 1: Study the contribution of cohesin mutations to hematopoiesis and AML
Aim 2: Examine the connection between loss cohesin or CTCF and trisomy 21
Aim 3: Elucidate the molecular mechanism for cohesin and CTCF involvement in progression to DS-AMKL

U54CA193419 (O'Halloran/Licht) 05/19/15 – 04/30/20 0.49 Calendar
NIH/NCI $46,680
Grant officer: Barbara Liesenfeld
9609 Medical Center Drive
West Tower, 2nd floor
Rockville MD 20850
Telephone: (204) 276-6294
Fax: (240) 276-7861
Email: liesenfb@mail.nih.gov
Spatio-Temporal Organization of Chromatin and Information Transfer in Cancer
Project 3: Mechanisms of Nuclei Chromosomes and Chromatin in Cancer (Marko)
The goal of Project 3 will be a comprehensive study of how chromatin and chromosomes are remodeled in cancer cells relative to normal cells, and its research will be tightly linked to Project 1 and Project 2 via parallel studies of the same cell lines, use of the physical assays developed in Project 3, and through cooperation on development of siRNA and CRISPR methodologies.
Specific aim: To study how chromatin and chromosomes are remodeled in cancer cells relative to normal cells
Role: Co-Investigator

Changes:
The following projects have been awarded - please see above for further details

- R6480-15 (Crispino) 09/01/15-08/31/17
  Leukemia and Lymphoma Society
  MLN8237, an Aurora A kinase inhibitor, for the treatment of myeloid malignancies

- 9001-16 (Crispino) 10/01/15 - 09/30/18
  Leukemia & Lymphoma Society
  Identification of pathways that promote transformation of the MPNs to AML

- Research Grant (Crispino) 07/1/16 - 06/30/18
  Rally Foundation
  DYRK1A is a novel target in B cell leukemia

- Research Grant (Crispino) 10/01/15 – 09/30/17
  Heartland Blood Centers
  Role of cohesion mutations in Acute Myelogenous Leukemia

- U54CA193419 (O'Halloran/Licht) 05/19/15 – 04/30/20
  NIH/NCI
  Spatio-Temporal Organization of Chromatin and Information Transfer in Cancer
  Role: Co-Investigator

The following projects have ended:

- 6429-13 (Crispino) 10/01/12 - 09/30/15 0.60 Calendar
  Translational Research Grant $180,018
  The Leukemia and Lymphoma Society
  Novel therapies for pre-B cell ALL
  The goal of this proposal is to perform pre-clinical studies to support development of novel therapies for pediatric B-ALL

- C-037 (Lahn) 02/01/13 - 01/31/15 0.12 Calendar
  Chicago Biomedical Consortium $37,425
  Dissecting cis verus trans regulation of gene expression using in vitro transcription
  The goal of this project is to develop a method capable of distinguishing whether the silent state of a gene is rendered causally by diffusible factors acting in trans of the gene or chromatin modifications acting in cis of the gene.
  Specific Aim: To develop an experimental system capable of dissecting cis versus trans regulation of gene expression.
  Role: Co – PI

What other organizations were involved as partners?
Nothing to Report.

SPECIAL REPORTING REQUIREMENTS
Not Applicable

APPENDICES – please see following pages

1. Manuscript: Dual deficiency of mDia1 and miR-146a in an age-related inflammatory bone marrow microenvironment induces ineffective erythropoiesis that phenocopies del(5q) MDS. Yang Mei[1], Ashley A Basiorka[2], Baobing Zhao[1], Jing Yang[1], Alan List[2,3], and Peng Ji.
2. Figures

Dual deficiency of mDia1 and miR-146a in an age-related inflammatory bone marrow microenvironment induces ineffective erythropoiesis that phenocopies del(5q) MDS

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Abstract

Myelodysplastic syndromes (MDS) are a group of age-related clonal hematologic diseases characterized by anemia, neutropenia, and thromobocytopenia. The mechanisms of anemia in MDS are unclear, which is due to the heterogeneity of MDS involving many cytogenetic and molecular abnormalities. Using a mouse genetic approach, here we show that dual deficiency of *mDia1* and *miR-146a*, two genes located at chromosome 5q that is commonly deleted in MDS, causes an aged-related anemia and ineffective erythropoiesis that closely phenocopies human MDS. Various stages of bone marrow erythropoiesis were dramatically affected in aged mDia1/miR-146a double knockout mice, which induced a massive splenomegaly with potent extramedullary erythropoiesis. Using different transplantation assays, we demonstrate that the ageing bone marrow microenvironment is important for the development of ineffective erythropoiesis. Consistent with the roles of mDia1 and miR-146a in regulating innate immune responses, the serum levels of TNFα and IL-6 were significantly elevated in old mDia1/miR-146a double knockout mice. Damage-associated molecular pattern molecules (DAMPs), whose levels increase in ageing microenvironment, induced TNFα and IL-6 upregulation in mDia1/miR-146a double knockout granulocytes and T cells. Mechanistically, we demonstrated that the anemia and ineffective erythropoiesis were independent of hepcidin expression in mDia1/miR-146a double knockout mice. Instead, pathologic levels of TNFα and IL-6 inhibit erythroid colony formation and differentially affect terminal erythropoiesis through reactive oxygen species-induced caspase-3 activation and cell apoptosis. Our study underscores the dual roles of the ageing microenvironment and cytogenetic abnormalities in the pathogenesis of ineffective erythropoiesis in MDS.

Introduction

Myelodysplastic syndromes (MDS) are a group of age-related bone marrow diseases characterized by dysplastic or ineffective production of myeloid cells. Patients with MDS often have increased risk of developing acute myeloid leukemia (AML). Clinically, a great majority of patients with MDS show anemia caused by ineffective production of erythroid cells at different stages of erythropoiesis. The pathogenesis of ineffective erythropoiesis in MDS is unclear, which can be attributed to the heterogeneous nature of many cytogenetic and molecular abnormalities involved in the development of MDS. The most common cytogenetic defect in MDS is the heterozygous deletion of chromosome 5q (del(5q)). There are two common deleted regions (CDRs) on 5q: a distal locus that is commonly deleted in 5q- syndrome (sole 5q deletion) with good prognosis and a proximal locus deleted in patients with higher risk of MDS. Refractory anemia is a defining feature of del(5q) MDS. Recent studies have shown that haploinsufficiency of *Rps14* on the distal locus of the CDRs blocks erythroid differentiation through upregulation of p53 and its downstream genes *S100a8* and *S100a9* (Lindsley and Ebert, 2013; Schneider et al., 2016). Whether deficiencies of other genes on chromosome 5q are involved in ineffective erythropoiesis is unknown.

Growing evidence reveals that dysfunction of innate immune responses is also involved in the pathogenesis of del(5q) MDS. Concurrent loss of miR-145 and miR-146a, which are located at the distal region of 5q, leads to the dysplastic phenotype in megakaryocytes by the upregulation of their downstream target tumor necrosis factor receptor-associated factor-6 (TRAF6)(Starczynowski et al., 2010a). Subsequent studies using miR-146a knockout mice show that miR-146a serves as a brake on inflammation

and regulates myeloproliferation and oncogenic transformation (Boldin et al., 2011). Our previous work reveals that mDia1, flanked by the two CDRs on 5q, was significantly decreased in del(5q) MDS CD34$^+$ cells. mDia1 heterozygous and knockout mice developed age-related neutropenia and myeloid dysplasia mimicking human MDS(Keerthivasan et al., 2014). Mechanistically, CD14 was aberrantly overexpressed on granulocytes, leading to a hypersensitive innate immune response to lipopolysaccharide (LPS) stimuli. A more recent study illustrates that loss of another 5q gene, *Tifab*, alters hematopoiesis through derepression of Toll-like receptor-TRAF6 pathway, leading to ineffective hematopoiesis and cytopenia (Varney et al., 2015). In these studies, leukopenia, including neutropenia, is commonly observed, which demonstrates that deregulation of the innate immune signaling is involved in myeloid dysplasia. However, anemia is mild or not observed in these models, indicating that either loss of these genes are not essential for the development of ineffective erythropoiesis, or combined deficiencies, as those in del(5q) MDS, are required for the clinical manifestation of anemia.

In this study, we generate a mouse model with concurrent deletion of mDia1 and miR-146a, two 5q genes involved in the repression of TLR-TRAF6 pathway. Mice with mDia1/miR-146a double knockout showed age-related anemia and ineffective erythropoiesis. We demonstrate that loss of mDia1 and miR-146a does not significantly affect erythropoiesis on their own. The ineffective erythropoiesis evident in these mice is rather caused by the upregulation of proinflammatory cytokines in a non-cell autonomous manner. We further reveal the distinct roles of TNFα and IL-6, two major proinflammatory cytokines abundantly secreted in these mice, in various stages of

erythropoiesis. Our study underlies the significance of aberrant innate immune signaling in the pathogenesis of ineffective erythropoiesis and anemia in del(5q) MDS.

Methods

Antibodies and chemicals

Carrier free recombinant mouse TNFα (Cat # 575202) and IL-6 (Catalog # 300-327P) were purchased from BioLegend and GEMINI respectively. PE-CD44 (IM7), APC-TER119, PE-CD71, and eFluor450-CD45.1 were purchased from eBiosciences. APC-CD45.2 was purchased from BioLegend. Other materials were described in detail elsewhere (Mei et al., 2016; Keerthivasan et al., 2014). Lipopolysaccharide (LPS) from Escherichia Coli 055:B5 was purchased from Sigma. The preparation, storage and chronic intraperitoneal injection of LPS were described previously (Keerthivasan et al., 2014).

Mice

miR-146a deficient mice were purchased from The Jackson Laboratory. mDia1 knockout mice were described previously (Keerthivasan et al., 2014). The mDia1 and miR-146a knockout mice were crossed to obtain mDia1/miR-146a double heterozygous mice. The four groups of littermates with different genotypes in this study were obtained through mDia1/miR146a double heterozygotes breeding. The congenic mice that carry the CD45.1 antigen (B6-LY-5.2/Cr, strain code: 564) were purchased from Charles River. All the experiments were conducted in accordance with the Guide for the Care and Use of

Laboratory Animals and were approved by the Institutional Animal Care and Use Committees at Northwestern University.

Bone marrow transplantation

Bone marrow transplantation was performed as described previously (Keerthivasan et al., 2014). Briefly, lethally irradiated (1000 rad) donor mice were injected retro-orbitally with approximately two million bone marrow mononuclear cells after removing red blood cells with ACK lysis buffer (Thermo Fisher Scientific, Cat # A1049201). The recipient mice were then supplied with antibiotic water and transferred to regular cages after 3 weeks. Complete blood cell counts (Hemavet 950, Drew Scientific) and flow cytometric analysis (BD FACSCanto II) were performed at different time points after transplantation to assess engraftment.

RNA extraction and quantitative real time-PCR

RNA isolation, complementary DNA synthesis and quantitative real time-PCR were performed as previously described (Mei et al., 2016). Primer sequences for mouse Hamp gene encoding Hepcidin: forward 5'- GCCTGTCTCCTGCTTCTCCT-3'; reverse 5'- GCTCTGTAGTCTGTCTCATCTGTT-3'. TNFα: forward 5'-GACAGTGACCTGGACTGTGG-3'; reverse 5'-TGAGACAGAGGCAACCTGAC-3'. IL-6: forward 5'-AGTCCGGAGAGGGAGAACAG-3'; reverse 5'-ATTTCCACGATTTCCCAGAG-3'. 18s rRNA: forward 5'-GCAATTATTCCCCATGAACG-3'; reverse 5'- GGCCTCACTAAACCATCCAA-3'.

In vitro erythroid differentiation and flow cytometric analysis

In vitro erythroid differentiation with bone marrow-derived lineage negative cells and flow cytometric analysis of bone marrow cells, splenocytes and peripheral blood were previously described (Keerthivasan et al., 2014). In brief, the bone marrow cells were flushed out from the tibia and femur using a syringe with 27.5G needle, passed through a 40μm cell strainer, and re-suspended in 15mL PBS. The cell concentration was measured by Z1 Particle and Cell Counter from Beckman. The absolute number of different cell populations was calculated by multiplying the bone marrow cell number with frequency acquired by flow cytometry. Lineage depletion was achieved by using the biotin mouse lineage panel (Cat # 559971, BD Pharmingen) according to the manufacturer's protocol. The lineage negative cells were then cultured in erythropoietin-containing medium with or without TNFα or IL-6 for 1-3 days. *In vitro* erythroid cell differentiation and enucleation were examined by staining the cells with Ter119, CD71 and Hoechst 33342 followed by flow cytometry as previously described (Zhao et al., 2014).

Peripheral blood was resuspended in red blood cell (RBC) lysis buffer (eBioscience, Cat # 00-4333-57) for 5 minutes on ice. Immediately after incubation, the RBC lysed cells were washed with ice cold PBS and passed through a 40μm cell strainer. Single cell suspensions of splenocytes were obtained by homogenization using the frosted ends of the slides and passing through 40μm cell strainer. All the cells were then labeled with appropriate antibodies for flow cytometry analysis.

CM-H2DCFDA (Cat#C6827) and CellROX® Orange Reagent (C10443) were purchased from ThermoFisher Scientific to detect the reactive oxygen species (ROS) levels in the cells. Active Caspase-3 level in the cells were assayed by SR-FLICA™ Caspase 3 & 7 Assay Kit (Cat # 931) from ImmunoChemistry Technologies. The assays were performed according to manufacturer's protocol.

In vitro PAMPs and DAMPs treatment

To prepare the damage-associated molecular patterns (DAMPs), total bone marrow cells from 6-8 weeks old mice were suspended in 1 ml cell culture-grade water and lysed by three freeze/thaw cycles. Granulocytes ($Gr1^+/Mac^+$) and T cells ($CD3\varepsilon^+$) from bone marrow or spleen were positively purified using the biotin mouse lineage panel. The purified cells were exposed to freeze-thaw cell lysates containing DAMPs at 1:20 for 2 h or subjected to 1 μg/ml LPS as pathogen-associated molecular pattern (PAMPs) for 1 h. Expression of TNFα and IL-6 mRNA levels in the cells was evaluated with a quantitative real-time PCR assay.

Colony Forming Cell (CFC) Assay

For colony-forming cell assay, total bone marrow cells from C57BL/6 mice were plated in triplicate in methylcellulose medium (Methocult M03234, StemCell Technologies) supplemented with 3 U/ml erythropoietin in the presence or absence of different concentrations of TNFα or IL-6 following the manufacturer's instructions. The CFU-erythroid (CFU-E) colonies were scored at 2-3 days with an inverted microscope (Olympus, CKX31).

Histology stains

Mouse sternum and spleen were fixed in 10% neutral-buffered formalin overnight. The samples were then embedded in paraffin and processed for hematoxylin and eosin (H&E) staining provided by Mouse Histology and Phenotyping Laboratory at Northwestern University. Peripheral Smear and Wright Giemsa – MayGrunwald staining were previously descried (Keerthivasan et al., 2014).

Statistical Analysis

Results presented in this study are expressed as mean ± SEM unless otherwise indicated. The statistical analysis was performed with Student's t test using GraphPad Prism version 6.0 software. Survival curve was compiled using Kaplan-Meier function of Prism software, and the significance was assessed using the Log-rank (Mantel-Cox) test. $P<0.05$ was considered statistically significant.

Results

Dual deficiency of mDia1 and miR-146a causes age-related anemia in mice.

To characterize the extent of pathologically activated innate immune responses on the development of anemia in del(5q) MDS, we crossed the mDia1 knockout mice with miR-146a knockout mice and generated double-heterozygous offspring. The mDia1/miR-146a double knockout (hereafter DKO), single knockouts, and double wild type (DWT) control littermates were further produced through double-heterozygous breeding. We monitored the complete blood counts in these mice over one year. Consistent with our previous reports, mDia1 KO mice showed no anemia and miR-146a KO mice had mild anemia compared to the DWT control mice (Keerthivasan et al., 2014; Boldin et al., 2011). In comparison, the DKO mice developed significant age-related anemia starting at 7 months, which worsened with aging (**Figure 1A**). The DKO mice also displayed thrombocytopenia compared to single KO and DWT control mice. Both DKO and mDia1 single KO mice developed neutropenia (**Figure 1A**), which is consistent with previous findings (Keerthivasan et al., 2014). Morphologic examination of the peripheral blood in the aged DKO mice showed severe anemia with anisopoikilocytosis including hypochromatic cells, Howell-Jolly bodies, and increased polychromasia (**Figure 1B**). Consistently, the reticulocyte count was dramatically increased in the aged DKO mice compared to wild type, mDia1 KO, or miR-146a KO littermate controls (**Figure 1C**). The DKO mice also exhibited increased lethality around one year old, likely due to a life-threatening anemia (**Figure 1D**).

We next examined the bone marrow of the one-year of mice to determine the cause of anemia. Gross observation showed pale bone and bone marrow cell aspirate from DKO

mice and to a lesser extent from miR-146a knockout mice (**Figure 2A**). Consistent with these findings, the total bone marrow cells in DKO mice were significantly decreased (**Figure 2B**). We reasoned that the pale appearance of the DKO bone marrow was due to the decreased erythroid population. Accordingly, Ter119 (a maturing erythroid marker) positive erythroid cells were significantly decreased in DKO mice compared to wild type and single knockout littermate controls, confirming our hypothesis (**Figure 2C**). The dramatic decrease of the erythroid population was accompanied by an increase in the relative percentage of Gr1 and Mac1 double positive myeloid cells (**Figure 2D**). However, the absolute number of myeloid cells was decreased in the bone marrow of DKO mice, albeit to a lesser extent than the decrease observed in the erythroid population (**Figure 2E**).

To further dissect aberrancies in erythroid cell differentiation, we next performed a flow cytometric analysis using CD44 and forward scatter to divide the erythroid cells into proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts, orthochromatic erythroblasts, reticulocytes, and mature red blood cells, which represent the least differentiated to enucleated mature forms, respectively (Liu et al., 2013). The percentage of nucleated erythroblasts in the aged DKO bone marrow was significantly decreased compared to littermate controls (**Figure 2F**). miR-146a KO mice also showed slightly decreased percentage of nucleated erythroblasts (**Figure 2G**). When the absolute numbers of erythroblasts were calculated from leg bones, aged miR-146a knockout mice showed a significant decrease, and erythroblasts from aged DKO mice further decreased compared to miR-146a knockout mice (**Figure 2H**). Consistent with the flow cytometric results, histological examination of the bone marrow from the old DKO mice

showed marked hypoplastic erythropoiesis with near absence of the erythroid population (**Figure 2I**).

Unlike patients with MDS, mice with anemia often show compensatory erythropoiesis evidenced by splenomegaly (An et al., 2015). As expected, the old DKO mouse showed a dramatically increased spleen size compared to wild type or single knockout littermate controls (**Figure 3A and B**). Previous data illustrates that under stressful conditions, splenic erythropoiesis undergoes BMP4-mediated promotion of development that is distinct from steady-state bone marrow erythropoiesis (Harandi et al., 2010). Consistent with this and with the dramatically ineffective erythropoiesis in aged DKO mice, the percentage of splenic Ter119 positive erythroid cells increased in DKO mice (**Figure 3C**). The Gr1/Mac1 double positive myeloid population also slightly increased in these mice, possibly compensating the decreased myeloid population in the bone marrow. The percentages of B cells and T cells were significantly reduced (**Figure 3C**). We next examined different stages of terminal erythropoiesis in the spleen. Unlike the bone marrow, terminal erythropoiesis in the spleen did not show defects in various stages of differentiation (**Figure 3D**), These data suggest that ineffective erythropoiesis in the bone marrow is compensated by splenic stress erythropoiesis, likely protecting the DKO mice from early death induced by severe anemia.

Age-related bone marrow microenvironment contributes to the development of anemia.

Both mDia1 and miR-146a knockout mice show age-related increased secretion of pro-inflammatory cytokines, including TNF-α and IL-6 (Keerthivasan et al., 2014;

Starczynowski et al., 2010b; Boldin et al., 2011). To determine whether the defects of erythroid population are cell intrinsic or due to the inflammatory microenvironment, we performed two sets of transplantation experiments. In these experiments, bone marrow mononuclear cells from old (> 1-year old) DKO mice or age matched DWT littermate controls were transplanted into either young (2-month old) or old (> 1-year old) recipient mice. Complete blood counts were tested at different time points following bone marrow transplantation and the survival of the transplanted mice was analyzed (**Figure 4A**). The young recipient mice showed no significant differences in red cell indices between DKO and DWT transplanted mice until 6 months post-transplantation when red blood cell count, hemoglobin, and hematocrit began declining (**Figure 4B**). The age when anemia manifested in these transplanted mice was similar to that of the regular DKO mice (**Figure 1A**), implicating an age-related microenvironment in the development of anemia. Similar to the non-transplanted aged DKO mice, these transplanted mice exhibited neutropenia early post-transplantation due to the cell-intrinsic effect of mDia1 deficiency, as well as worsening thrombocytopenia (**Figure 4C**). The mortality between these two groups of transplanted mice was similar to those of the regular DWT and DKO mice (data not shown).

In contrast to the gradual development of anemia in the young recipient mice transplanted with old DKO bone marrow cells, when we transplanted old DWT or DKO bone marrow cells to the old (> 1 year of age) recipient mice, the old recipient mice transplanted with old DKO bone marrow cells exhibited prominent anemia within 2 months post-transplantation (**Figure 4D**). Bone marrow analysis of the old recipient mice from both sets of the transplantation experiments showed ineffective erythropoiesis at different stages of red cell development similar to the non-transplanted old DKO mice

(**Figure 4E**). When these mice were followed for survival, we found that old recipient mice transplanted with old DKO bone marrow cells exhibit rapid lethality compared to the old DWT bone marrow transplanted controls (**Figure 4F**). Taken together, these results indicate that the age-related bone marrow microenvironment plays an important role in the development of ineffective erythropoiesis and anemia.

Chronic injection of lipopolysaccharide accelerates the development of ineffective erythropoiesis in young mDia1/miR-146a double knockout mice.

We previously reported that loss of mDia1 causes an aberrant overexpression of Toll-like receptor 4 (TLR4) adapter protein CD14 on granulocytes, which over-sensitizes innate immune signaling to lipopolysaccharide (LPS) challenge (Keerthivasan et al., 2014). miR-146a knockout mice also displayed a hypersensitive innate immune response to LPS mainly due to the derepression of TLR downstream transducers TRAF6 (Boldin et al., 2011; Yang et al., 2012). Since both mDia1 and miR-146a are involved in innate immune signaling, we asked whether chronic immune stimulation would accelerate the development of ineffective erythropoiesis. To this end, we injected low doses of LPS chronically in young (6-7 weeks old) DKO mice and age-matched littermate controls. Unlike the untreated DKO mice that developed anemia and ineffective erythropoiesis at 7 months of age (**Figure 1**), the LPS treated mice started to show anemia, thrombocytopenia, and neutropenia at 4 months (**Figure 5A**). The absolute number of Ter119 positive erythroid cells was also significantly decreased at 4 months of age (**Figure 5B**). Similar to the old DKO mice, erythroblasts at various developmental stages were decreased compared to the DWT and single knockout controls (**Figure 5C**). These mice also showed prominent splenomegaly, compensating

for ineffective erythropoiesis within the bone marrow (**Figure 5D**). These results support the role of an inflammatory bone marrow microenvironment in the development of ineffective erythropoiesis and anemia in the context of concurrent loss of mDia1 and miR-146a.

Proinflammatory cytokines are over-produced in aged mDia1/miR-146a double knockout mice.

Given the significance of the bone marrow microenvironment in the development of anemia, we next examined the levels of proinflammatory cytokines in old DKO mice and littermate controls. As expected, serum levels of both IL-6 and TNFα were significantly increased in old DKO mice compared to age-matched DWT and single knockout controls (**Figure 6A**). The ageing bone marrow microenvironment is composed of increasing amounts of damage associated molecular patterns (DAMPs), which are potent inducers of proinflammatory cytokines production (Shaw et al., 2013). We previously demonstrated that mDia1 deficient Gr1/Mac1 positive granulocytes over-produced TNFα and IL-6 when treated with LPS (Keerthivasan et al., 2014). A similar phenotype was also observed in miR-146a knockout mice (Boldin et al., 2011; Zhao et al., 2013). To analyze whether treatment of DAMPs could also induce the over-production of proinflammatory cytokines in old DKO mice *in vitro*, we purified Gr1 and Mac1 double positive granulocytes from the bone marrow and spleen of young DKO mice and littermate controls. These cells were treated with DAMPs prepared through repetitive freeze thaw cycles of wild-type bone marrow cells. Similar to LPS, treatment with DAMPs induced over-production of TNF-α and IL-6 in the bone marrow- and spleen-derived myeloid cells from each group of mice, with levels particularly high in DKO cells

(**Figure 6B**). TNFα and IL-6 levels were also dramatically higher in CD3ε positive T cells from the spleen of DKO mice compared to the controls (**Figure 6C**). These results indicate that bone marrow microenvironment-mediated ineffective erythropoiesis in DKO mice could be related to the over-secretion of proinflammatory cytokines.

TNFα and IL-6 negatively affect erythropoiesis through different mechanisms.

Proinflammatory cytokines are known to be involved in anemia of chronic disease (ACD) through upregulation of liver hepcidin, which limits the availability of iron to the developing erythroblasts (Lee et al, 2005; Pietrangelo et al, 2007)(Andrews, 2004). However, the increased cytokine levels did not induce hepcidin unregulation in hepatocytes from aged DKO mice compared to control mice (**Figure 7A**), indicating that ineffective erythropoiesis in aged DKO mice was likely hepcidin-independent. Next, we investigated whether the proinflammatory cytokines TNFα and IL-6 directly affect erythropoiesis *in vitro*. We first purified the lineage negative bone marrow cells from old (> 1 year age) DWT and DKO mice and cultured them in erythropoietin-containing medium for three days. No statistically significant differences were observed in their differentiation, as quantified by CD71 and Ter119 double positive cells (**Figure 7B**). Similar findings were obtained from young DKO bone marrow lineage negative cells (data not shown), supporting the rationale that ineffective erythropoiesis is non-cell autonomous. Therefore, we used DWT bone marrow to determine how proinflammatory cytokines affect various stages of erythropoiesis. Previous studies have demonstrated inhibitory roles of proinflammatory cytokines in erythropoiesis (Weiss and Goodnough, 2005). However, the mechanisms cytokine-mediated inhibition of erythropoiesis, the stages at which the inhibitory effects are conferred, and whether there are any

differences between TNFα and IL-6 in inducing ineffective erythropoiesis, are still unknown.

To answer these questions, we purified bone marrow mononuclear cells from young DWT bone marrow and cultured the cells in methocellulose with erythropoietin and increasing amounts of TNFα and IL-6. Both TNFα and IL-6 induced a dose-dependent decrease in Colony-Forming Unit- Erythroid (CFU-E) colony formation (**Figure 7C**), demonstrating that increasing levels of both of these cytokines affect the early stage of erythropoiesis. After the CFU-E stage, the erythroid cells undergo terminal erythropoiesis that can be monitored by flow cytometry. We next determined the differentiation of DWT bone marrow lineage negative cells cultured with erythropoietin and increasing amount of TNFα and IL-6. These cytokines did not significantly affect cell differentiation, as measured by Ter119 positive cells. Instead, high levels of both TNFα and IL-6 induced increased Ter119 positive cells on day 1 (**Figure 7D**), possibly contributing to the depletion of erythroid progenitor cells.

We next analyzed whether increasing amounts of TNFα and IL-6 could induce cell death during terminal erythropoiesis. Flow cytometric analysis using annexin V and propodium iodine (PI) revealed that IL-6 induced a dose-dependent increase in annexin V positive apoptotic cells at different stages of terminal erythropoiesis. TNFα-mediated apoptosis (annexin V positive) was only observed on day 3 (**Figure 7E**). TNFα also induced mild necrosis (annexin V negative and PI positive) on day 2 and 3 whereas IL-6 treated cells showed no increased necrosis compared to the vehicle controls (**Figure 7F**).

Reactive oxygen species (ROS) induced by proinflammatory cytokines are known to promote apoptosis (Circu and Aw, 2010). Our previous report demonstrated that ROS is at the highest level in the early stages of terminal erythropoiesis, which corresponds to day 1 of the erythroid differentiation culture system (Zhao et al., 2016). ROS levels gradually decreased on day 2 and 3 in control cells (**Figure 7G**). Administration of IL-6 led to a dose-dependent increase of ROS beyond the physiologic level, which was most prominent on day 1. In contrast, TNFα treated cells did not show increased ROS levels (**Figure 7G**). Consistent with the role of ROS in the activation of caspase-3, IL-6 treated cells exhibited a dose-dependent increase of active caspase-3, correlating with their apoptotic cell death. No increased activation of caspase-3 was observed in TNFα-treated cells (**Figure 7H**). We also tested the cell cycle profiles of IL-6 or TNFα-treated cells. In the early stage of terminal erythropoiesis, many erythroid cells stay in S and G2/M phases, consistent with their rapid differentiation and proliferation. Cells in the late stage of terminal erythropoiesis exit the cell cycle and mostly are in the G1 phase (Ji et al., 2010). The percentage of S and G2/M phases were slightly increased with the treatment of IL-6 and TNFα on day 1 (**Figure 7I**), which could contribute to the increased number of Ter119+ cells (**Figure 7D**). Taken together, these results demonstrate the TNFα and IL-6 negatively affect erythropoiesis through different mechanisms. Both cytokines compromise CFU-E colony formation at higher levels. In addition, IL-6 also induces ROS-mediated activation of caspase-3 and apoptosis, which is not observed with TNFα.

Increased ROS and apoptosis in erythroid cells *in vivo* in mDia1/miR-146 double knockout mice

We next determine whether ROS and apoptosis were increased *in vivo* in erythroid cells from DKO mice. Similar to *in vitro* cytokine treated erythroid cells, ROS levels were markedly upregulated beyond the physiologic level at different stages of terminal erythropoiesis in DKO mice compared to DWT controls (**Figure 8A**). Annexin V positive apoptotic cells were also significantly increased in the Ter119 positive erythroid cells from DKO mice compared to DWT or single knockouts (**Figure 8B-C**). As expected, different stages of terminal erythroblasts showed increased apoptotic cells, as measured by activation of caspase-3 (**Figure 8D**).

Lenalidomide has been FDA approved for the treatment of del(5q) MDS given its immunomodulatory effects and ability to induce impressive hematologic and cytogenetic responses (Crane and List, 2005; Gañán-Gómez et al., 2015). To determine whether suppression of inflammatory cytokines would rescue ineffective erythropoiesis, we treated the DKO mice with lenalidomide. To this end, 3-month-old DKO mice and littermate control DWT mice were treated with lenalidomide (10 mg/kg) every 20 days for 5 consecutive months. After treatment, bone marrow erythroid cells at different developmental stages were analyzed. Although no significant effects were observed in lenalidomide-treated DWT cells, lenalidomide treatment rescued the decreased erythroblast population in the DKO mice, especially in polychromatic and orthochromatic stages of terminal erythropoiesis (**Figure 8E**).

Discussion

In this study, we revealed the significant roles of the aging bone marrow microenvironment and deletion of human chromosome 5q genes in the pathogenesis of ineffective erythropoiesis in MDS. Dual deficiency of miR-146a and mDia1 induces a hypersensitivity to the stimulation of the innate immune system, which leads to an age-related pathologic increase in proinflammatory cytokines causing ineffective erythropoiesis and anemia. Stimulation of young mDia1/miR-146a double knockout mice with chronic LPS injection accelerates the development of ineffective erythropoiesis and anemia, which further underlines the significance of hypersentitive innate immune responses in the pathogenesis of anemia in del(5q) MDS.

Deletions of 5q genes in del(5q) MDS are uniformly hemizygous. Although other genetic abnormalities in the retained 5q alleles have not been detected so far, recent studies indicate that many genes on the retained 5q alleles are epigenetically silenced. In fact, epigenetic dysregulation is commonly seen in MDS leading to the loss of function of many tumor suppressor proteins (Khan et al., 2013). Therefore, we employed the mDia1 and miR-146a double knockout mouse model instead of the double heterozygous mice in this study. This approach also makes it convenient to compare the current data with previous reports using miR-146 knockout mice to study MDS.

Our results reveal that the decreased bone marrow erythroid population in miR-146a/mDia1 double knockout mice and miR-146a knockout mice causes a proportional

increase of the $Gr1^+/Mac1^+$ myeloid population. However, the absolute number of these cells is not increased. In miR-146a/mDia1 double knockout bone marrow, the $Gr1^+/Mac1^+$ myeloid cells are actually decreased slightly. Reductions in the bone marrow erythroid and myeloid populations induce a compensatory increase of these lineages in the spleen. Therefore, the proportionally increased $Gr1^+/Mac1^+$ myeloid population in the bone marrow and their increase in absolute number in the spleen may give a false impression of a myeloproliferative phenotype, as previously reported in miR-146 knockout mice. Here we propose that the mDia1/miR-146a double knockout mice phenocopy ineffective erythropoiesis and represent a model of anemia that is commonly seen MDS. However, the possibility of a myeloproliferative phenotype cannot be ruled out entirely. As these mice die around one year of age because of severe anemia, a further analysis of other myeloid neoplasms cannot take place.

Anemia is one of the hallmark features of del(5q) MDS. Several studies have revealed that genes that are commonly deleted on chromosome 5q, such as RPS14, are critical for the development of anemia. RPS14 heterozygosity induces p53-dependent apoptosis and cell cycle arrest in erythroid cells. RPS14 haploinsufficient late stage erythroblasts also overexpress proteins involved in innate immune function, such as S100A8 and S100A9, which negatively affect terminal erythropoiesis *in vitro* and *in vivo*. Our data reveal a non-cell autonomous role of activated innate immunity in the pathogenesis of ineffective erythropoiesis. We show that proinflammatory cytokines TNFα and IL-6 are predominantly produced by granulocytes in the bone marrow and T cells in the spleen in mDia1/miR-146a double knockout mice in response to the PAMPs (LPS) or DAMPs, which are mostly identified in the ageing microenvironment. This is further confirmed through transplantation experiments in which young recipient mice transplanted with old

DKO bone marrow cells do not exhibit anemia or ineffective erythropoiesis until the mice are aged. These results demonstrate that the erythroblasts from mDia1/miR-146a double knockout mice are functionally intact and the ineffective erythropoiesis observed in these mice may be attributable to pathologic levels of proinflammatory cytokines.

TNFα and IL-6 are known to inhibit erythropoiesis. However, the detailed mechanisms of how different cytokines are involved in ineffective erythropoiesis are unclear. One of the most commonly involved pathways in inflammation-related anemia is cytokine-mediated hepcidin upregulation in the liver, which negatively affects the availability of iron to the developing erythroblasts. However, hepcidin is not increased in our DKO mouse model. Instead, our study revealed direct but distinct roles of TNFα and IL-6 in the pathogenesis of ineffective erythropoiesis. Both cytokines blocked the erythroid colony formation. They also promoted the differentiation of the early stage of terminal erythropoiesis after the CFU-E stage. In addition, IL-6 also induced potent upregulation of reactive oxygen species and caspase-3 mediated apoptosis whereas TNFα only caused mild necrosis. These results are also consistent with reported clinical observations that IL-6 and caspase-3 is upregulated in MDS patients with refractory anemia.

IL-6 is upregulated in miR-146a knockout mice through increased expression of TRAF6, a well-known target of miR-146a. Our previous study shows that IL-6 and TNFα are also prone to be upregulated in mDia1 deficient mice when the mice are subject to innate immune stimulation, which is due to the increased expression of CD14 on granulocytes. Here we show that the combined deficiency of miR-146a and mDia1 leads to a

dramatically increased secretion of these cytokines, especially IL-6. This increase is most prominent in old mice, which is consistent with the age-related changes in the bone marrow microenvironment. The level of IL-6 correlates with the level of ineffective erythropoiesis. In miR-146a/mDia1 double knockout mice, IL-6 is nearly four-fold higher than miR-146a knockout mice and 20-30-fold higher than the wild type or mDia1 knockout mice. Correspondingly, bone marrow erythroid cells decrease significantly in miR-146a knockout mice and almost completely absent in miR-146a/mDia1 double knockout mice. These results are consistent with a more severe effect of IL-6 in erythropoiesis.

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Authorship

Contribution: Y. M. designed and performed the research, analyzed, interpreted the data, and wrote the manuscript; A.A.B. B.B. Z., J. Y. performed the experiments; A.L. interpreted the data. P.J. designed, analyzed and interpreted the data, and wrote the manuscript.

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Figure Legends

Figure 1. mDia1/miR-146a double knockout mice develop age-related anemia.

(A) Complete blood count of different time points of mDia1/miR-146a double wild type (DWT), mDia1 or miR-146a single knockout (KO), and double knockout (DKO) mice. DWT, n=6; miR-146a KO, n=6; mDia1 KO, n=5; DKO, n=7. (B-C) Wright-Giemsa stains of peripheral blood smear from indicated mice at one year old. Blue arrow: polychromatic cell. Black arrows: hypochromatic cells. Scale bars: 10 μm. The relative percentages of reticulocytes in peripheral blood were quantified from at least 800 cells and shown in C, n=3 mice in each group. Data are shown as mean ± standard error of the mean (SEM). (D) Kaplan-Meier survival analysis of the indicated mice. DWT, n=14; miR-146a KO, n=8; mDia1 KO, n=6; DKO, n=11. Both males and females were included.

Figure 2, Bone marrow ineffective erythropoiesis in aged mDia1/miR-146a double knockout mice.

(A) Representative photographs of bone and bone marrow cell suspension from indicated mice at one year old. (B-E) Flow cytometric analysis of bone marrow cells from indicated mice at one year old. Total bone marrow cells (B), absolute Ter119^{+} erythroid cells (C), relative parentages of Gr1/Mac1 double positive granulocytes (D), and the absolute number of Gr1/Mac1 double positive granulocytes (E) from femur and tibia were presented. DWT, n=6; miR-146a KO, n=4; mDia1 KO, n=5; DKO, n=5. (F-H) The Ter119^{+} erythroid cells in C were further analyzed by CD44 levels and forward scatter to define various developmental stages of erythroblasts (Pro-: proerythroblasts, Baso-: basophilic erythroblasts, Poly-: polychromatic erythroblasts, Ortho-: orthochromatic erythroblasts, reticulocytes (Retic-) and mature red blood cells (RBC) in F. Quantification of the percentages and absolute cell numbers of erythroblasts at each stage of

differentiation were presented in G and H, respectively. DWT, n=6; miR-146a KO, n=4; mDia1 KO, n=5; DKO, n=5. (I) H&E stains of bone marrow sections of indicated mice. Arrows indicate erythroblasts. Scale bar: 10 μm.

Figure 3, Aged mDia1/miR-146a double knockout mice develop significant splenomegaly and extramedullary erythropoiesis.

(A-B) Representative photograph of spleens from indicated mice more than one year old in A. The spleen size versus body weight was quantified in B. DWT, n=15; miR-146a KO, n=7; mDia1 KO, n=8; DKO, n=8. (C) Flow cytometric analysis of erythroid cells (Ter119$^+$), granulocytes (Gr1$^+$/Mac1$^+$), B cells (B220$^+$), and T cells (CD4$^+$ and CD8α$^+$) in the spleens from indicated mice (> 1 y old). DWT, n=6; miR-146a KO, n=4; mDia1 KO, n=5; DKO, n=5. (D) The Ter119$^+$ erythroid cells in C were further analyzed by CD44 and forward scatter assayed as in Figure 2F. DWT, n=6; miR-146a KO, n=4; mDia1 KO, n=5; DKO, n=5.

Figure 4. The aging bone marrow microenvironment plays an important role in the development of anemia and ineffective erythropoiesis.

(A) Schematic overview of the bone marrow transplantation experiments. (B-C) Post-transplant complete blood counts at the indicated time of recipient mice (2 m old when transplanted) transplanted with bone marrow cells from old DWT or DKO mice (> 1 y old). N=5 in each group. (D) Post-transplant red cell indices at the indicated time of recipient mice (>1 y old when transplanted) transplanted with bone marrow cells from old DWT or DKO mice (>1 y old). N=10 in each group. (E) The bone marrow Ter119$^+$ erythroid cells from old recipient mice (>1 y old) from both sets of transplantation experiments were further analyzed by CD44 levels and forward scatter to define various developmental

stages of erythroblasts as in Figure 2F. N=3 in each group. (F) Kaplan-Meier survival analysis of the recipient mice in D. N=18 in DWT; N=16 in DKO.

Figure 5, Chronic LPS challenge accelerates the development of anemia and ineffective erythropoiesis in mDia1/miR-146a double knockout mice

(A) Complete blood counts of indicated 6-7 week-old young mice challenged with chronic LPS (2 μg per gram of body weight, once every three weeks) injection for 3 months. DWT, n=17; miR-146a KO, n=10; mDia1 KO, n=9; DKO, n=12. (B) Total bone marrow cell and erythroid cell counts from indicated LPS-treated mice after 3 months. (C) Bone marrow erythroid cells at different developmental stages from B were analyzed by flow cytometic analysis with Ter119 and CD44 as in Figure 2F. N=6 in each group. (D) Quantification of spleen versus body weight of the indicated mice. N=6 in each group.

Figure 6, Proinflammatory cytokines are over-produced in aged mDia1/miR-146a double knockout mice.

(A) Serum IL-6 and TNFα from indicated mice (> 1 y old) were assayed by ELISA. DWT, n=6; miR-146a KO, n=4; mDia1 KO, n=5; DKO, n=6. (B-C) Gr1/Mac1 double positive granulocytes (B) or Splenic $CD3\varepsilon^+$ T cells (C) from indicated mice (> 1 y old) were purified and challenged with low dose of LPS (1 ug/ml for 1 h) and/or damage-associated molecular pattern proteins (DAMPs) (1:20 for 2 h). The relative mRNA levels of TNFα and IL-6 were determinate by real-time PCR analysis.

Figure 7, Proinflammatory cytokines directly attenuate erythropoiesis *in vitro*

(A) Quantification of hepcidin mRNA levels in hepatocytes by real-time PCR. N=5 in each group. (B) Bone marrow lineage negative cells (Lin^-) from indicated mice were

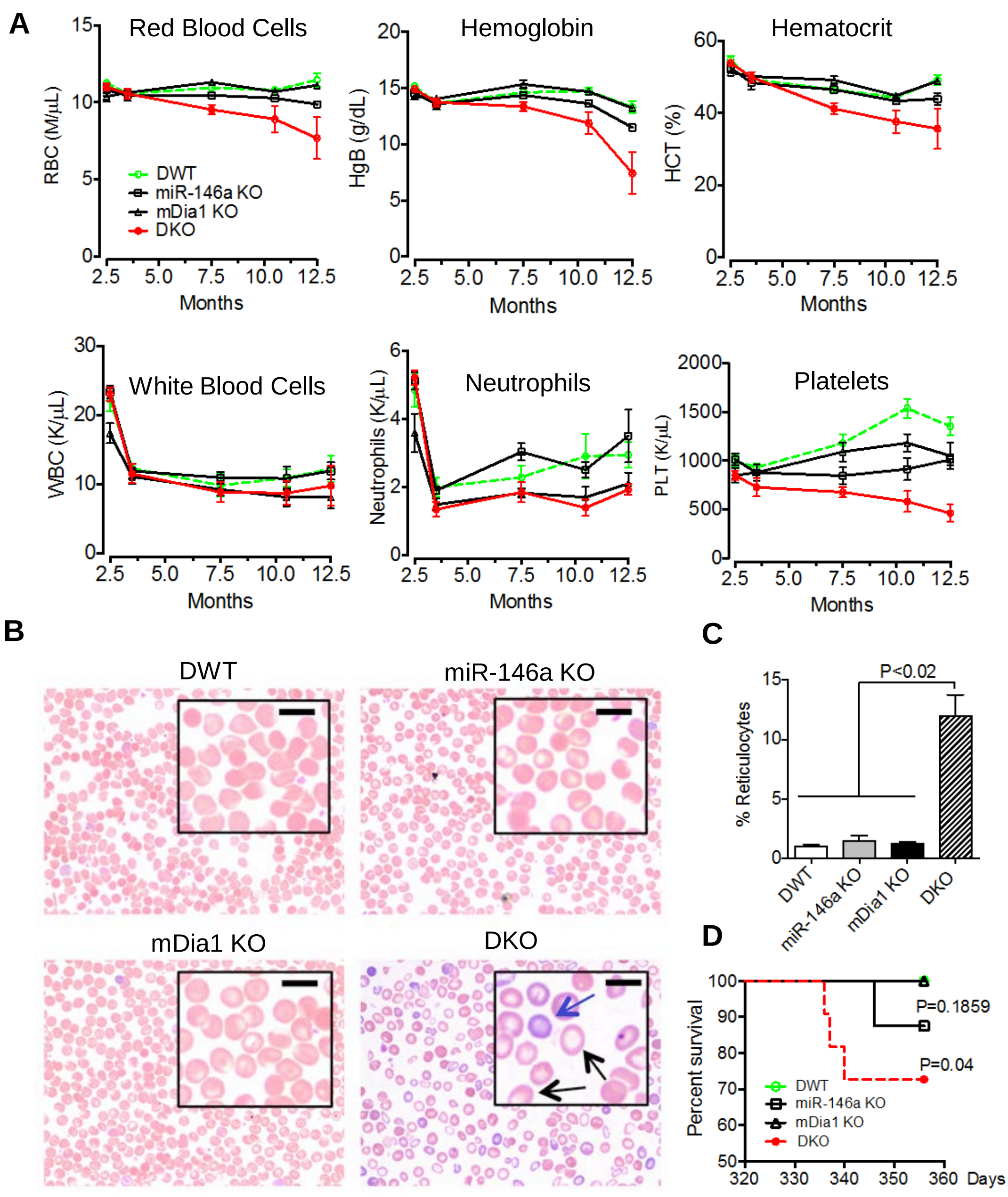
cultured in erythroid differentiation medium containing 2U/ml erythropoietin. Cell differentiation was analyzed by flow cytometric analysis comparing the expression of transferrin receptor CD71 and Ter119. (C) CFU-E colony formation assay of bone marrow cells cultured in methylcellulose medium with 3U/ml erythropoietin and increasing amounts of TNFα and IL-6. (D-F) Lin^- cells from C57BL/6 mice were purified and cultured in erythroid differentiation medium in the presence of increasing amounts of IL-6 and TNFα (50,100, and 200 ng/mL) for 3 days. The Ter119 positive cells were quantified in D. Annexin V positive cells in the Ter119+ erythroid cells were quantified in E. Necrotic cells in F were determined as annexin V negative and PI positive. (G-H) The ROS and active caspase-3 levels were assayed by flow cytometry in Ter119 positive cells from D, and were shown in G and H respectively. Intracellular ROS levels were analyzed and presented as the mean fluorescence intensity (MFI) of dichlorodihydrofluorescein (DCF), an oxidation product of CM-H2DCFDA. (I) Cell cycle analysis was performed in Ter119 positive cells in D. The percentages of the cycling cells (S/G2/M phases) are shown. All the Data are shown as mean ± SEM and representative data from 3 independent experiments. NS: not significant. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

Figure 8, Increased reactive oxygen species and apoptosis in the erythroid cells from old mDia1/miR-146a double knockout mice.

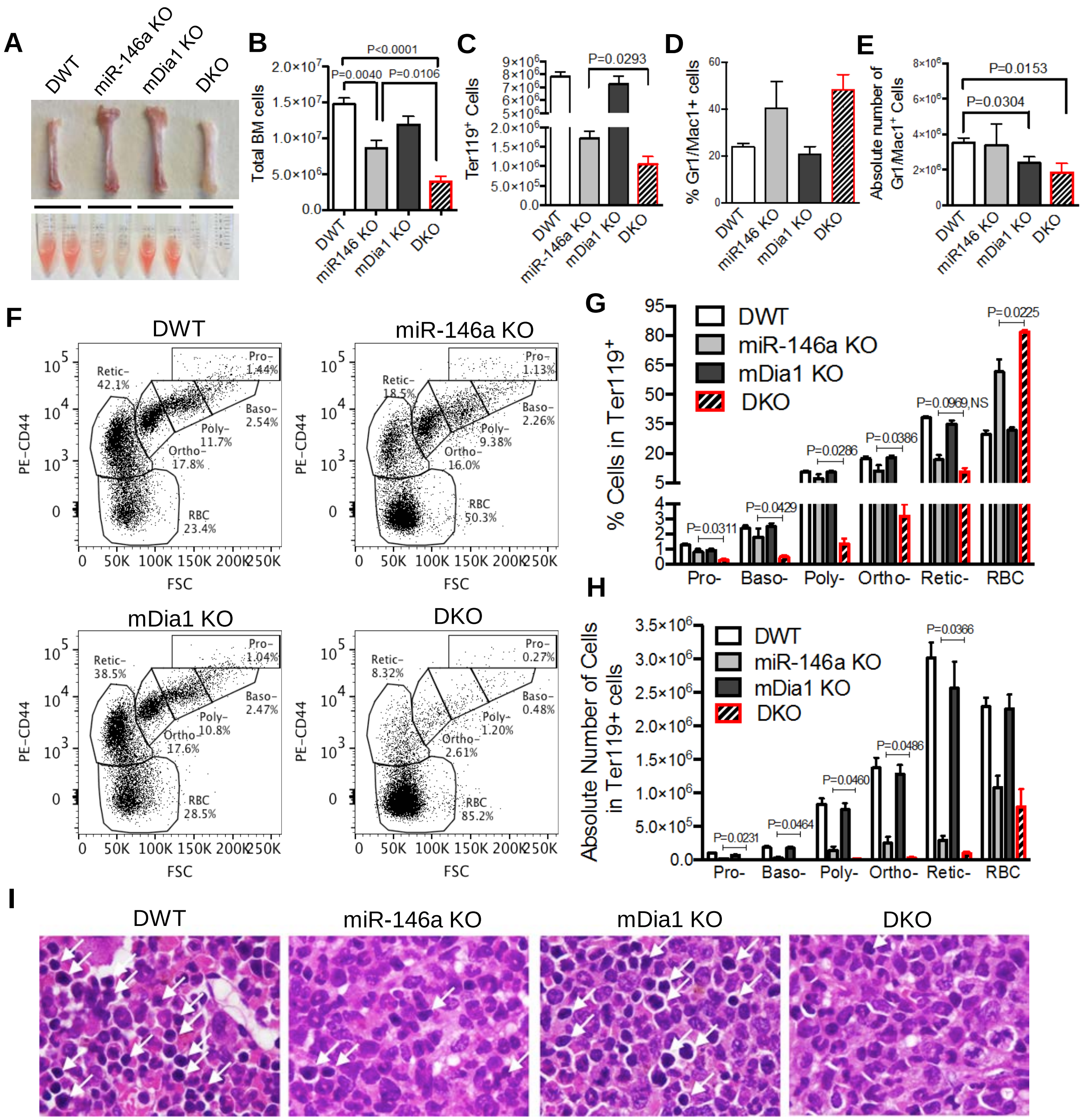
(A) Mean percentage and mean fluorescence intensity (MFI) of ROS positive cells were analyzed at different populations of the erythroblasts and ungated bone marrow erythroid populations in old mDia1/miR-146a DWT or DKO mice (> 1 y old). DWT, n=8; DKO, n=5. (B-C) Flow cytometric analysis of apoptosis in $Ter119^+$ bone marrow erythroblasts from indicated mice (> 1 y old). The percentages of annexin V positive cells were shown in C. (D) Mean percentages of apoptotic cells in different populations and

ungated bone marrow erythroblasts from indicated mice (> 1 y old). DWT, n=7; DKO, n=4. (E) Three-month old DWT and DKO young mice were treated with lenalidomide (10mg/kg) every 20 days for 5 consecutive months. Bone marrow terminal erythropoiesis was analyzed by flow cytometry using Ter119 and CD44 stains as Figure 2F. N=3 in each group.

Mei et al. Figure 1



Mei et al. Figure 2



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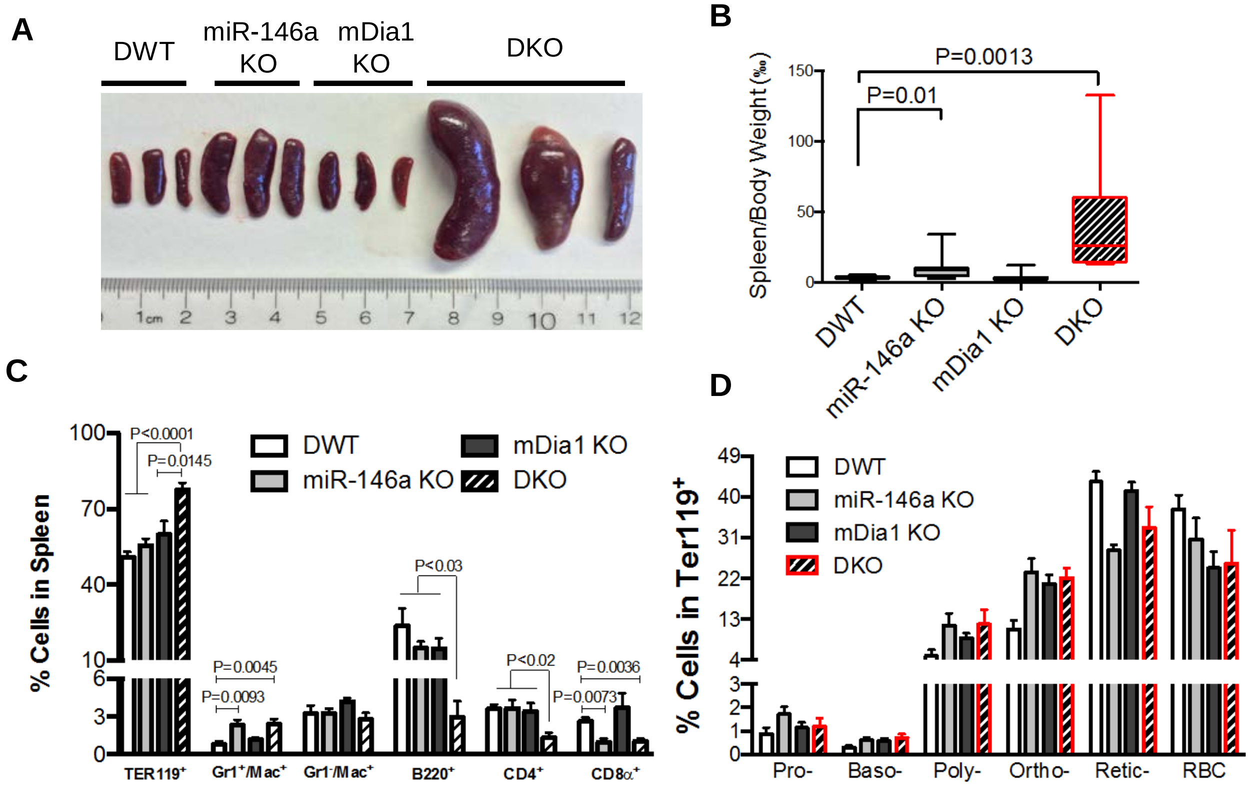

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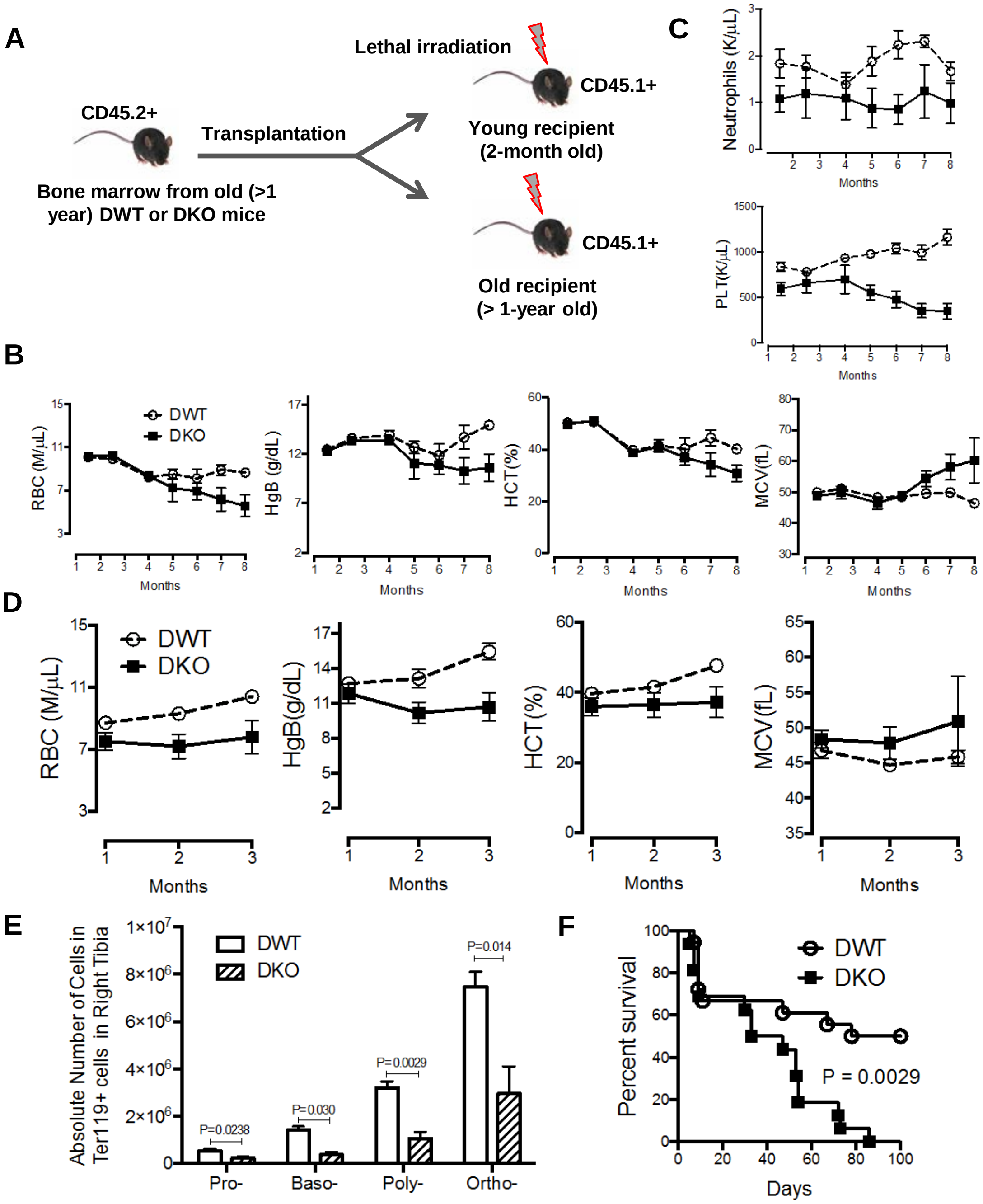

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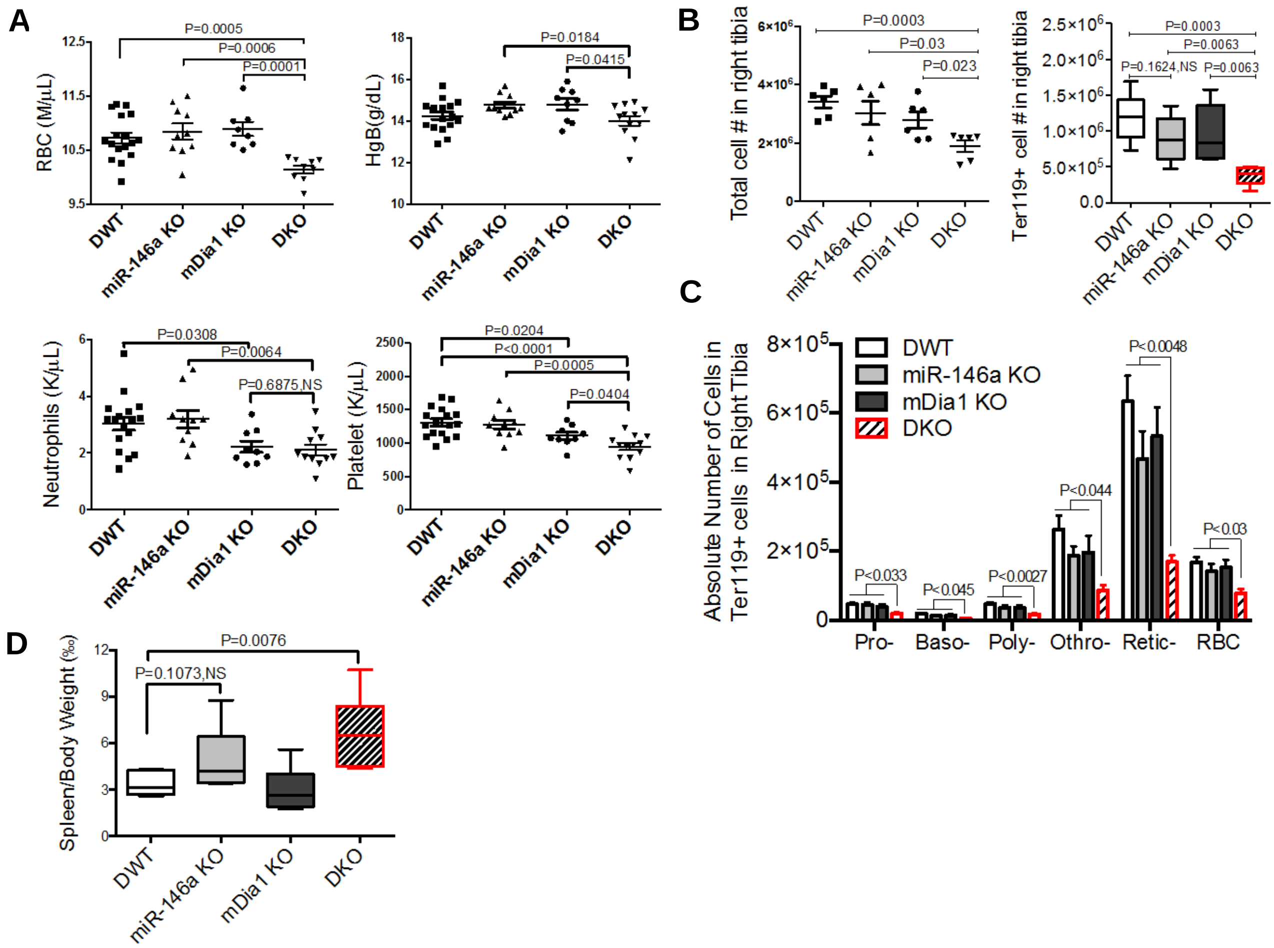

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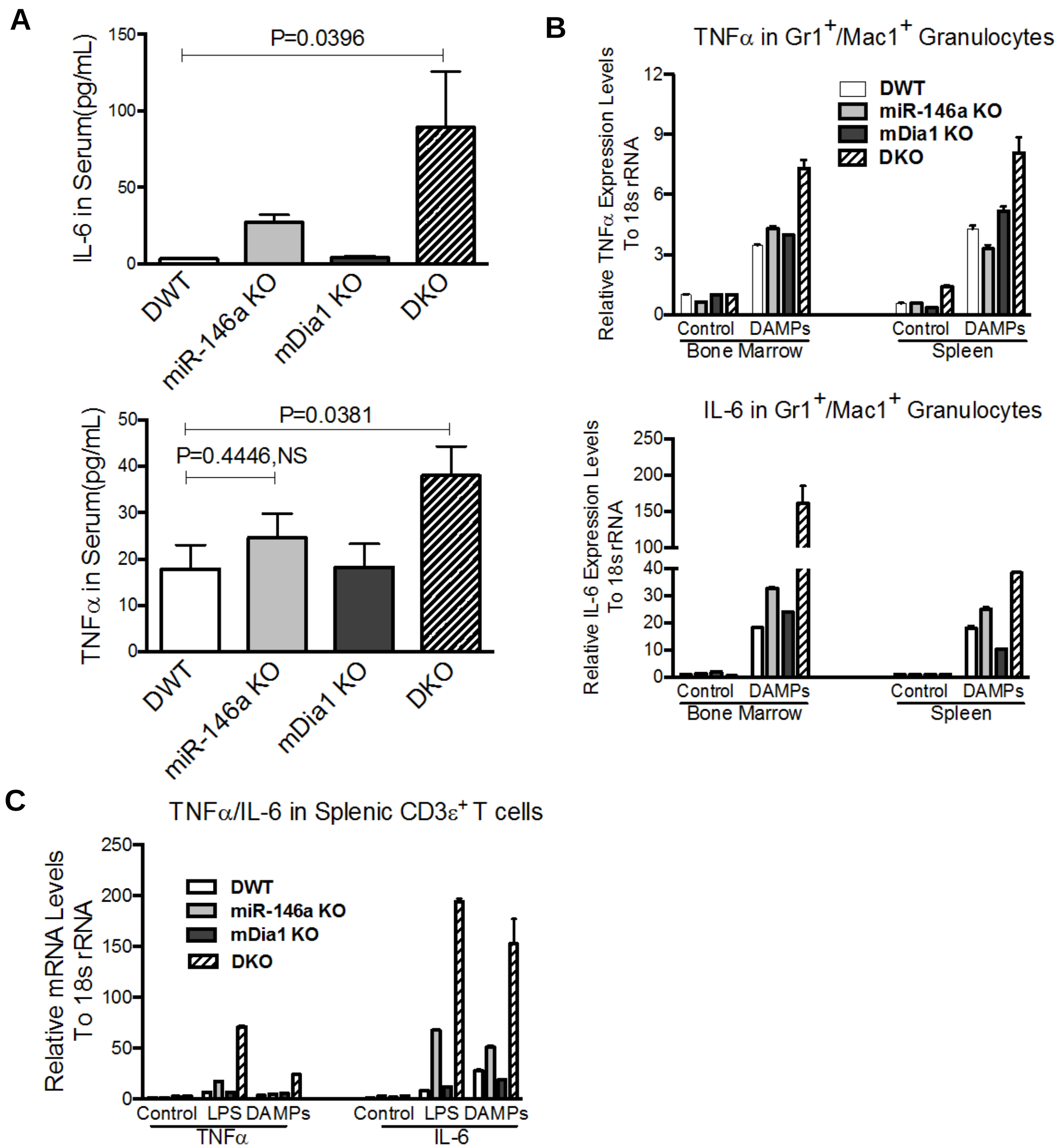

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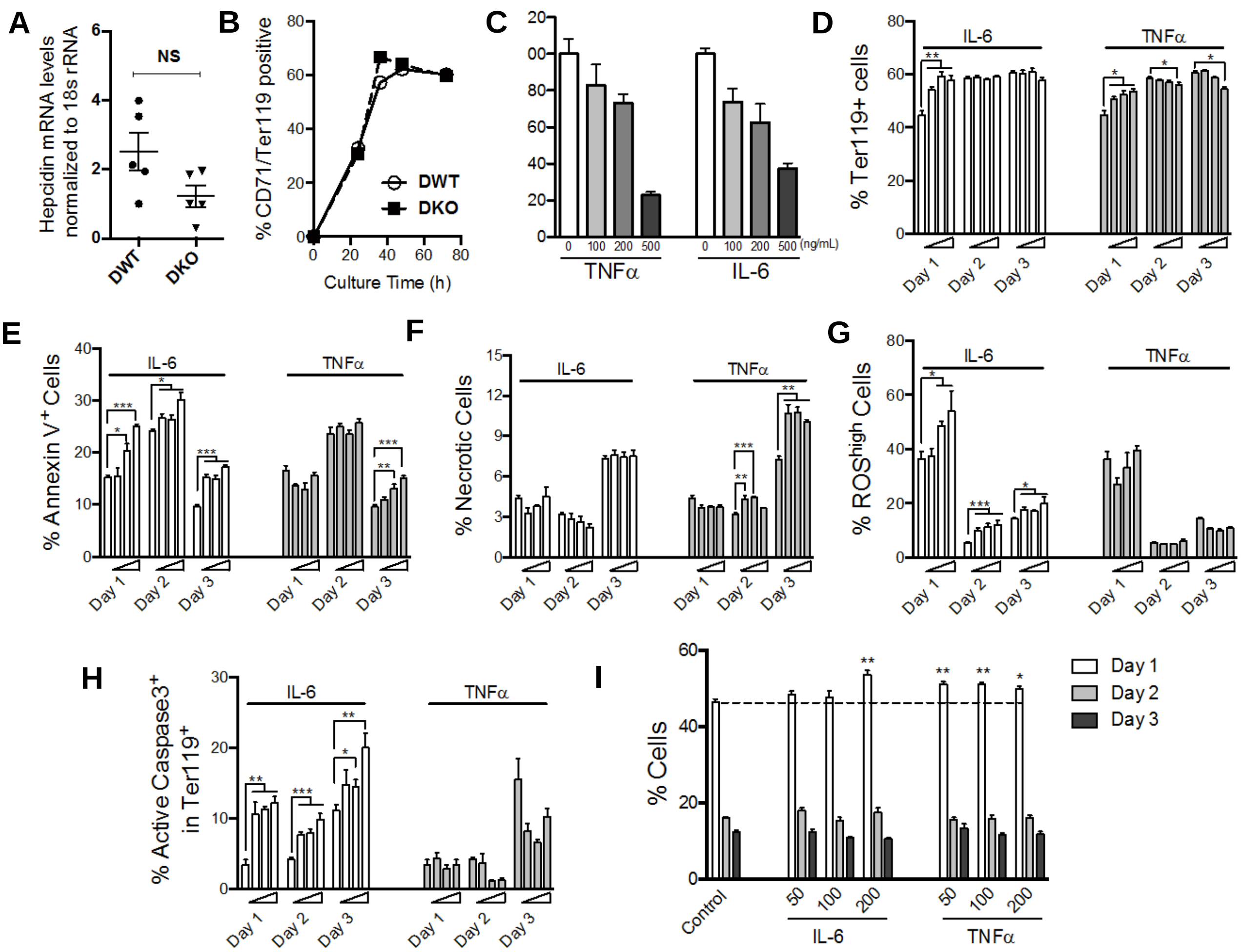

Mei et al. Figure 8